Protein Digestibility and Relevance to Allergenicity

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In January 2001 a Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Consultation Committee on Allergenicity of Foods Derived from Biotechnology published a report outlining in detail an approach for assessing the allergenic potential of novel proteins. One component of this decision tree is a determination of whether the protein of interest is resistant to proteolytic digestion. Although these *in vitro* methodologies have been useful, the correlation between resistance to proteolysis and allergenic activity is not absolute. Two views and highlights of supporting research regarding the relationship of resistance to digestion and allergenicity are presented in this article. *Key words:* allergenicity, protein stability, protein structure, proteolytic digestion, safety assessment, sequence homology, simulated gastric fluid. *Environ Health Perspect* 111:1122–1124 (2003). doi:10.1289/ehp.5812 available via *http://dx.doi.org/* [Online 19 December 2002]

In January 2001, a Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Consultation Committee on Allergenicity of Foods Derived from Biotechnology (FAO/WHO 2001) published a report outlining in detail an approach for assessing the allergenic potential of novel proteins. As described elsewhere, this approach takes the form of a hierarchical decision tree, one component of which is consideration of the resistance of proteins to proteolytic digestion (digestion in simulated gastric or intestinal fluids or by pepsin). The inclusion of this parameter in current safety assessment paradigms is based largely upon the studies of Astwood et al. (1996) and others (Becker 1997; Besler et al. 2001; Burks et al. 1992b; Taylor et al. 1987; Taylor and Lehrer 1996), who found an association between plant proteins that displayed resistance to digestion in a simulated gastric fluid (SGF) and allergenic activity. It must be acknowledged, however, that the stability of most food allergens has not been determined. Although this method is attractive insofar as it does not necessitate the use of experimental animals and requires only modest amounts of the test protein, it is generally acknowledged that the correlation between resistance to proteolysis and allergenic activity is not absolute, and that, for example, not all stable proteins are able to induce allergic sensitization. Moreover, there is also some debate about whether the association between stability to digestion and allergenic potential necessarily reflects that for the induction of sensitization, proteins must be able to survive in the hostile gastrointestinal tract for a period of time sufficient to elicit an immune response. Researchers have speculated that the association between allergenicity and stability may alternatively, or additionally, be due to the way in which protein allergens are processed within cells for subsequent presentation to the immune system. Notwithstanding uncertainties about the mechanistic basis for a correlation between allergenicity and resistance to proteolysis, the work presented here presents various viewpoints of the utility of digestion studies in protein allergy safety assessments.

Experimental

Studies by Bannon and others in support of the use of* in vitro *digestibility assays in allergenicity assessment. Analysis of a variety of allergenic foods has resulted in identification of certain biochemical characteristics shared by many but not necessarily all food allergens (Stanley and Bannon 1999). One such characteristic is that they are relatively stable proteins and resistant to denaturation. This is thought to be an important characteristic because the longer significant portions of the protein remain intact the more likely it is to trigger an immune response (Astwood et al. 1996). The observation that many of the food allergens are proteins containing intramolecular disulfide bonds that may be important to their allergenicity (Lehrer et al. 1996) has led to the assumption that protein structure may be an important factor in the ability of an allergen to resist denaturation.

The pepsin digestibility assay was conceived as a means to determine the relative stability of a protein to the extremes of pH and pepsin protease encountered in the mammalian stomach and was originally developed and used as a method to assess amino acid bioavailability (Marquez and Lajolo 1981; Nielson 1988; Zikakis et al. 1977). The logic behind this test was that nutritionally desirable proteins tend to be rapidly digested and would be expected to have less opportunity to exert adverse health effects when consumed. This logic appears to have been confirmed, in part at least, for milk and wheat allergy. Buchanan and colleagues (Buchanan et al. 1997; del Val et al. 1999) reported that when structure of the major allergens from these foods is disrupted by reduction of disulfide bonds, the allergens were strikingly sensitive to pepsin digestion and lost their ability to elicit allergic reactions in previously sensitized dogs. Standardization of the assay conditions (such as pepsin concentration, pH, and temperature) has been described in the U.S. Pharmacopia (1990) and is sometimes referred to as SGF. The assay was not meant to mimic precisely the fate of proteins in *in vivo* conditions but rather to evaluate the susceptibility of the protein to digestion under fixed conditions *in vitro*. The purpose is to provide information that, in conjunction with other evidence, would be useful in predicting whether a dietary protein may become a food allergen. Therefore, the relationship of the resistance to digestion by pepsin and the likelihood a dietary protein is an allergen was identified and subsequently recommended by the U.S. Food and Drug Administration, U.S. Environmental Protection Agency, and U.S. Department of Agriculture (Department of Health and Human Services 1992) as a means of aiding the assessment of proteins added to commodity crops through biotechnology.

The digestive stability of the major allergens found in the most common allergenic foods were the first to be studied. The stability of some of the major allergens of peanut, soybean, egg, and milk relative to the stability of common nonallergenic food proteins was determined in the standard pepsin digestion assay (Astwood et al. 1996). Under the conditions described for SGF in this study, all food allergens were more resistant to pepsin hydrolysis than were common plant proteins. However, not all allergens from the most common allergenic foods were stable in the pepsin digestion assay for 60 min. Stability of the whole protein or fragments from the allergens tested ranged from 8 to 60

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min, whereas all nonallergen plant proteins tested did not survive in the pepsin digestion assay for more than 15 sec.

Since this initial report, there have been numerous studies repeating the pepsin digestion assay on these major food allergens and other food allergens (Besler et al. 2001). In general, the original findings that food allergens were stable to pepsin digestion relative to nonallergenic proteins were confirmed, but the length of time either the whole protein or fragments of the allergen were stable did not always agree. The most likely explanation for this quantitative difference is subtle changes in the pepsin digestibility assay or in the method by which the proteins of interest were detected. For example, changes in enzyme concentration, pH, protein purity, and method of detection could have substantial effects on the interpretation of any *in vitro* assay. In addition to these quantitative differences, some food allergens were unstable to pepsin digestion. One example is patatin (Sol t 1), an allergen of potato (Seppala et al. 1999) that is unstable in the pepsin digestion assay. For this reason, the International Life Sciences Institute has proposed a standardization process for the assay that will attempt to assess these variables so that results from different laboratories can be compared directly. Federal, academic, and industry laboratories from Europe, North America, and Japan will participate in this test, where pH (1.2/2.0), pepsin concentration, allergen purity, and method of detection have all been standardized.

With the objective of determining whether protein structure contributes to the ability of allergens to resist denaturation and digestions by enzymes encountered in the human gastrointestinal (GI) tract, a major peanut allergen, Ara h2, was studied. The Ara h2 peanut allergen is recognized by serum IgE from > 90% of patients with peanut allergy, thus establishing the importance of this protein in the etiology of the disease (Burks et al. 1992a, 1995). Ara h2 is resistant to acidic conditions and digestion with GI tract enzymes (Astwood et al. 1996). The linear IgE-binding epitopes of the Ara h2 allergen have been mapped using overlapping peptides and serum IgE from a population of patients known to be sensitized to peanuts. Ara h2 contains 10 IgE-binding epitopes detected with linear peptides representing the major epitopes recognized by serum from a peanut-sensitive patient population. Immunodominant IgE-binding epitopes were also determined from a population of peanut-sensitive patients for Ara h2. Ara h2 contained 3 epitopes (epitopes 3, 6, 7) recognized by serum IgE from the majority of patients tested and represented the majority of allergen-specific IgE found in these patients (Stanley et al. 1997).

Several methods have been used to gain a better understanding of the structural properties of Ara h2 that may contribute to its stability and allergenicity. The Ara h2 proteins do not form any higher order oligomeric structures with themselves but do contain eight cysteine residues that have the potential to form up to four disulfide bonds. To determine whether the disulfide bonds contribute to the secondary or tertiary structure of this protein, circular dichroism measurements were performed in the presence or absence of a reducing agent (dithiothreitol). Native or reduced Ara h2 was monitored either at the far (190–250 nm) or near (250–320 nm) ultraviolet (UV) ranges. The best estimates of secondary structure proportions obtained from the far UV data for the native Ara h2 were 18.2% of the molecule in α-helices, 54% in β-pleated sheets, and 27.7% in a random coil configuration. When the molecule was reduced, significant differences were found in secondary structure fractions. Reduced Ara h2 exhibited a secondary structure predominated by β-pleated sheet (82.3%), with the remainder of the molecule mostly in a random coil configuration.

The Ara h2 protein was exposed to proteases encountered in the GI tract to determine whether the native protein structure, as mediated by disulfide bonds, played any role in protecting it from degradation. Native Ara h2 protein was exposed to trypsin, chymotrypsin, or pepsin, either before or after the reduction of disulfide bonds, and then electrophoresed on 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The resulting peptides were visualized by staining with Coomassie blue. The native Ara h2 protein digested with these enzymes produced a 10-kDa protein fragment that was stable for the length of the experiment. In contrast, the Ara h2 protein that was first reduced and then digested with these enzymes did not produce any significant enzyme-resistant protein fragments and appeared to be much more susceptible to the action of the proteases when compared with the native protein.

To determine if the most protease-resistant Ara h2 fragments contained IgE-binding epitopes, the protein was exposed to chymotrypsin, and the reactions were electrophoresed on SDS-PAGE gels, blotted to nitrocellulose, and probed with serum IgE from a pool of peanut-sensitive patients. The 10-kDa protease-resistant peptide contained intact binding sites that could be recognized by IgE. Knowing that Ara h2 contains 10 IgE-binding sites evenly distributed along the linear sequence of the molecule (Stanley et al. 1997), these results suggest this fragment of Ara h2 contains multiple IgE-binding epitopes and survives digestion by the GI enzymes tested. The 10-kDa protease-resistant fragment was purified, and amino terminal sequencing was performed to identify what portion of the allergen this peptide represented. The amino acid sequence indicated the 10-kDa fragment begins at amino acid position 23 and contains about 90 amino acids. This portion of the Ara h2 protein contains IgE-binding epitopes 2–7 and six of eight of the cysteine residues. Interestingly, this fragment also contains 11 potential chymotrypsin cleavage sites.

These results demonstrate that protein structure plays an important role in the stability of this allergen to resist digestion and provide a link between food allergen structure, stability to digestion, and the immunodominant IgE-binding epitopes within a population of food-allergic individuals.

Studies by Fu and others that raise issues regarding use of* in vitro *digestibility assays for allergenicity assessment. Several recent investigations do not support the view that food allergens are necessarily more resistant to digestion than are nonallergenic proteins. Vieths et al. (1999) measured the digestibility of peanut and hazelnut allergens and found that although peanut proteins were stable, the native hazelnut allergens were susceptible to digestion. Yagami et al. (2000) showed that a number of vegetable food allergens degraded rapidly in SGF.

Kenna and Evans (2000) compared the digestibility of 17 food allergens with 24 proteins not normally associated with food allergy in SGF and found that even though 13 of the 17 food allergens were partially or completely stable for at least 60 min, 10 of the 24 nonallergenic proteins also were completely stable or formed peptide fragments that were stable for at least 60 min.

Our laboratory has compared the digestion stability of 17 food allergens and 18 nonallergenic proteins of similar cellular functions in SGF as well as in a simulated intestinal fluid (SIF) (Fu 2002; Fu et al. 2002). We found that the digestive stability of the food allergens tested varied greatly, ranging from 0 to 120 min. A similar range of SGF and SIF stability was observed among the nonallergenic proteins tested. These data suggest that food allergens may be more, equally, or less susceptible to SGF and SIF digestion than nonallergenic proteins of similar cellular functions, and that food allergens are not necessarily more resistant to digestion than are nonallergenic proteins. The implication of this criterion for allergenicity is that digestibility alone is not necessarily a reflection of the allergenic potential of a protein.

The relative allergenicity of a protein is described sometimes as a function of the number of individuals who develop sensitization or who display allergic disease. However, it must be acknowledged that the frequency

or prevalence of sensitization or clinical allergy may be a function of the nature, extent, and duration of exposure, rather than the inherent allergenic potency of the protein per se. Fuchs and Astwood (1996) compared the SGF digestibility of a selected group of egg, milk, and soybean allergens and suggested a correlation could be established between the digestive stability of food allergens and relative allergenicity. However, when additional food allergens were analyzed, it was found recently that the relative resistance to digestion did not necessarily correspond to high allergenicity (Fu et al. 2002). For example, ovalbumin, a major allergen, degraded within 5 min in SGF, whereas lysozyme, a minor egg allergen, was stable for at least 60 min. α-Casein, a major milk allergen, degraded more rapidly than the minor milk allergen bovine serum albumin. These results suggest there may not be a clear correlation between the digestibility of an allergen and relative sensitizing potency. As a consequence, it would be inappropriate to rank allergenic activity on this basis alone.

The use of digestion stability as a criterion for protein allergenicity assessment also stems from the general belief that for a protein to elicit an allergic response, it must survive the acid and proteolytic environment of the human GI system to reach, and be absorbed through, the intestinal mucosa (Taylor et al. 1987). Digestive stability has therefore been considered a key prerequisite for food allergenicity (Metcalfe et al. 1996). However, few data are available in the literature to verify that the structural integrity of a protein is necessary for the initiation of sensitization.

Several issues should be addressed if digestibility is to be used as part of the safety assessment process for novel proteins. One issue is standardization of assay conditions. A protein could appear stable or unstable in SGF (or SIF), depending on the relative amounts of enzyme and test protein used (Astwood et al. 1996; Fu et al. 2002). Some studies of effects of proteolytic digestion on allergenicity have used comparatively low ratios (by weight) of enzyme:protein, ranging from 0.1 to 0.01 (Asselin et al. 1989; Marquez and Lajolo 1981; Maynard et al. 1997; Watanabe et al. 1990). However, *in vitro* digestion assays for the purposes of safety assessment tend to employ higher ratios ranging from 25 to 5,000 (Fu 2002; Fuchs et al. 1993; Noteborn et al. 1995; Reed et al. 1996).

The second issue is one of kinetics and data interpretation. Fuchs and Astwood (1996) showed that nine different proteins expressed in genetically modified plants were rapidly degraded within 30 sec in SGF compared with the > 2-min stability shown by allergens. However, others have employed different time frames for definition of stability. Momma et al. (1999) considered a soybean glycinin expressed in genetically engineered rice to be labile in SGF when the protein was digested within 30 min. Noteborn et al. (1995) concluded that Cry1Ab was labile to digestion in SGF, although a 15-kDa fragment was still present after 2 hr of pepsin digestion. There remains a need therefore to establish guidelines for the interpretation of digestion assay results.

Conclusion

The consensus of the workshop participants was that digestion stability alone should not be used for defining an unknown protein as an allergen. Available data suggest that stability to digestion may not be a universal, defining characteristic of food allergens. Part of the difficulty in interpreting data from *in vitro* pepsin digestion assays is that different labs utilize different assay conditions (pH, enzyme/protein ratios, detection methods; Fu 2002; Fuchs et al. 1993; Noteborn et al. 1995; Reed et al. 1996). The workshop participants agreed there was a need to harmonize assay conditions for measurement of protein digestion and for a consensus on the most appropriate approach to data interpretation.

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